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TITLE: The Influence of Primary Microenvironment on Prostate Cancer Osteoblastic Bone Lesion Development

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14. ABSTRACT
Loss of the stromal TGF-β signaling in the prostate has been shown to initiate prostate cancer (PCa), promote PCa progression, and facilitate the development of mixed osteoblastic/osteolytic bone lesions. However, the effects on bone lesions are found to be transient. We thus focused on delineating the context-dependent role of TGF-β signaling in the bone microenvironment effects on PCa bone lesions. Using genetic engineered mouse models, TGF-β signaling is cell-specifically knockout (KO) in the prostate fibroblasts and osteoblasts in the bone by *Col*^{cre}/*Tgfbr2* KO, or in the myeloid lineage cells, including osteoclasts in the bone by *LysM*^{cre}/*Tgfbr2* KO. Compared the PCa-induced bone lesions in the KO mice tibiae to the lesions in the Flox mice, we found that PC3-induced osteolytic bone lesions were significantly increased by *Col*^{cre}/*Tgfbr2* KO, but were significantly decreased by *LysM*^{cre}/*Tgfbr2* KO. Our findings suggested that osteoblastic TGF-β signaling inhibits PCa bone lesions development, but myeloid TGF-β signaling promotes PCa bone lesion development. We further found that basic FGF mediated the effect of increased PC3 bone lesions in *Col*^{cre}/*Tgfbr2* KO mice.

More exciting, we found LUCaP osteoblastic bone lesion development, if it happens, takes 4 additional weeks in *LysM*^{cre}/*Tgfbr2* KO mice, but not in *Col*^{cre}/*Tgfbr2* KO mice, relative to the control mice. These data suggested that *LysM*^{cre}/*Tgfbr2* KO sets up a bone microenvironment that promotes PCa dormancy. We further found that a critical piece of *LysM*^{cre}/*Tgfbr2* KO mouse bone microenvironment is the expression of claudin-19 in osteoblast in establishing a dormancy-permissive microenvironment for the PCa cells.

15. SUBJECT TERMS
TGF-β signaling, osteoblasts, osteoclasts, myeloid, osteolytic, osteoblastic, bone lesions, cytokines, bFGF

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Introduction:

The hypothesis of this proposal is that cytokines/chemokines regulated by stromal TGF-β signaling from the primary tumor microenvironment dictate prostate cancer (PCa) osteoblastic bone metastasis. We proposed to determine the contribution of prostate stromal TGF-β in PCa-induced osteoblastic bone lesion development and to determine the chemokines that induced by loss of TGF-β signaling mediate PCa blastic bone metastasis. However, we found that although loss of TGF-β responsiveness in prostate stromal cells do alter chemokine levels and facilitate the development of mixed osteoblastic/osteolytic bone lesions, these effects are transient (Li, 2012). These results suggest that it is the interactions between the dessminated PCa cells and the cells in the bone microenvironment determined the bone lesion development. More important, 70% of PCa patients have detectable tumor cells in the bone barrow at the time of initial diagnosis, and we know 13% of PCa patients each year after a long period of tumor dormancy, most of these patient (70-90%) have bone metastasis (Morgan, 2009; Siegel, 2014). Therefore, we are now focusing on determining how the bone microenvironment effects on PCa bone metastasis. We thus proposed that the bone microenvironment is the final determinant for PCa induced bone lesion; the influence of cell-specific TGF-β signaling in the bone microenvironment might be different on PCa bone lesion development; and bFGF is one of the downstream mediators for the cell-specific TGF-β signaling effect that can be targeted to reduce bone lesions.

To test these hypotheses, we have used inducible TGF-β type II receptor (TβRII) knockout (*Tgfbr2* KO) mouse models. *Col1*^{creERT}*/Tgfbr2* KO (*Col/Tgfbr2* KO), which have TGF-β signaling specific KO in fibroblasts and osteoblasts, and *LysM*^{cre}*/Tgfbr2* KO (*LysM/Tgfbr2* KO), which have TGF-β signaling specific KO in cells of myeloid lineage, such as osteoclast in the bone. These inducible mouse models were generated in *Rag2*$^{-/-}$ immunodeficient background, so that human PCa cells are able to xenografted into these mice. The mice were further crossed with *mT/mG* reporter mouse from Jackson Laboratory. Therefore the specific Cre expression can be determined through immunohistochemistry (IHC) using anti-GFP antibody in decalcified mouse bone tissues (Meng, 2015).

Keywords: TGF-β signaling, *Col*^{creERT}*/Tgfbr2* KO, *LysM*^{cre}*/Tgfbr2* KO, osteolytic, osteoblastic, osteoblast, osteoclast, bFGF, FGFR

Project Summary during the second year funding period of this award:

To determine the cell specific role of TGF-β signaling in the bone microenvironment effects on PCa bone lesions (months 12-18):

Task 1. Characterization of the above genetic-engineered mice: The bones are normal compared to the control floxed mice by x-ray and microCT analysis (Meng, 2015).

Task 2. Effects on PCa bone lesions:

2a. PC3 PCa-induced osteolytic bone lesion development was significantly promoted in the *Col*^{cre}*/Tgfbr2* KO mice relative to *Tgfbr2 Flox* (control) mice (**Figure 1**). The effect was repeated in DU145 cells (**Figure 2**). Further analysis of the bone lesions, we found the increased bone lesions were presented with increased angiogenesis and proliferation of the tumor cells in the bone, and osteoclastogenesis (**Figure 3**).

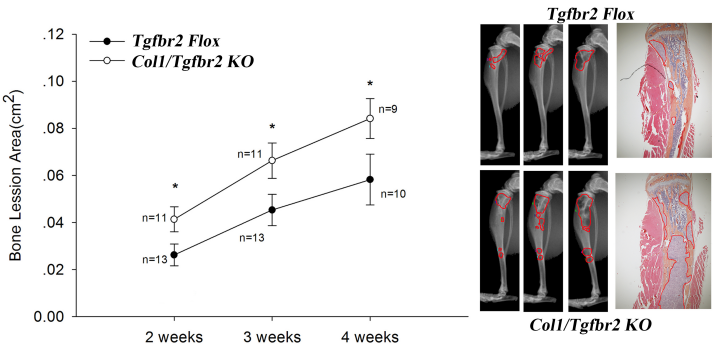

Figure 1. PC3 bone lesion development in *Col/Tgfbr2* KO mice relative to control mice.

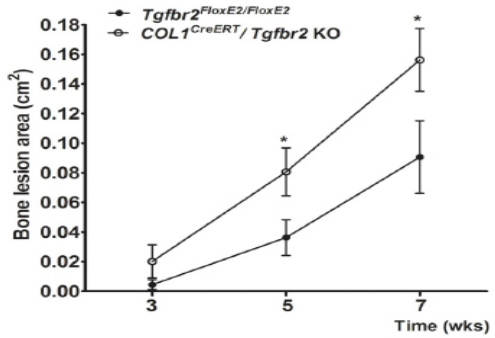

Figure 2. DU145 bone lesion development in *Col/Tgfbr2* KO mice relative to control mice.

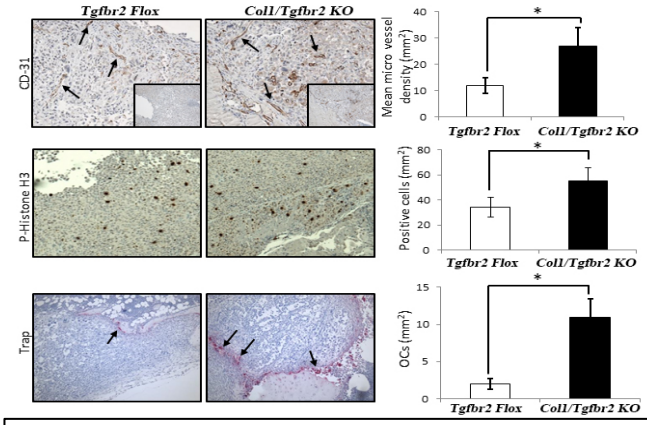

Figure 3. Histological analysis of PC3 bone lesions in *Col/Tgfbr2* KO and control mice relative to control mice.

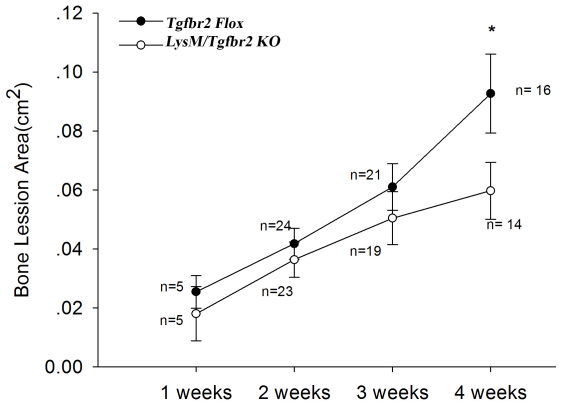

Figure 4. PC3 bone lesion development in *LysM/Tgfbr2* KO mice relative to control mice.

2b. *LysM*^{cre}*/Tgfbr2* KO significantly decreased PC3-induced bone lesions (**Figure 4**).

2c. Consistent with the data in PC3 cells, LUCaP tumor induced osteoblastic bone lesions were inhibited by *LysM/Tgfbr2 KO*. More importantly, the bone lesion development in *LysM/Tgfbr2 KO* (but not in the *Col/Tgfbr2* KO mouse), if it happens, takes additional 4 weeks to be detected; suggesting LysM/Tgfbr2 KO induced LUCaP PCa dormancy (**Figure 5**). Therefore, we propose loss of TGF-β signaling only in cells of the myeloid lineage creates a dormancy-permissive bone microenvironment for PCa cells.

Therefore, we conclude that TGF-β signaling in cells of the osteoblast lineage inhibits, but in cells of the myeloid lineage promotes PCa induced bone lesions.

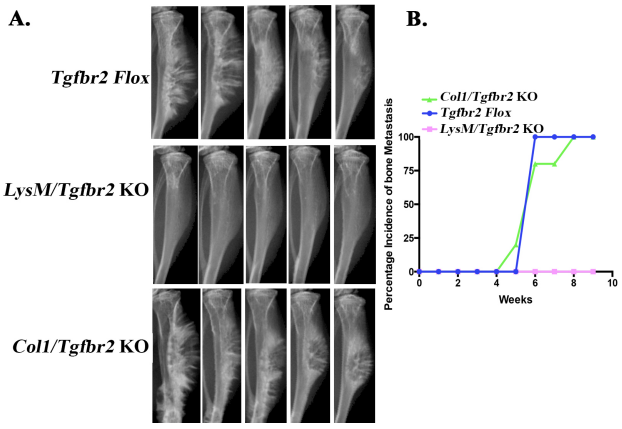

Figure 5. LUCaP bone lesion development in *LysM/Tgfbr2* KO, but not *Col/Tgfbr2* KO mice, if happens, takes four additional weeks, relative to control mice. **A.** Lesion pictures by X-ray from individual mouse. **B.** Bone lesion incidence. Note in this particular experiment, the mice were sacrificed at 9 weeks post tumor injections before lesions reached measurable size.

To determine the downstream mediator for cell specific role of TGF-β signaling in the bone microenvironment effects on PCa bone lesion devleopment (months 18-24):

Task 3. Downstream mediators of TGF-β signaling in cells of the osteoblast lineage for inhibiting PCa bone lesion development:

3a. Basic fibroblast growth factor (bFGF) in PC3 bone metastasis: bFGF was identified by cytokine array screening and confirmed to have increased expression at both mRNA level and protein level in PC3 induced tibiae of *Col/Tgfbr2* KO relative to control mice. IHC revealed that the bFGF was expressed mainly in osteoblasts and FGFR1 in PC3 cells, which were all increased in the lesions from *Col/Tgfbr2* KO mouse bone relative to control mouse (**Figure 6**).

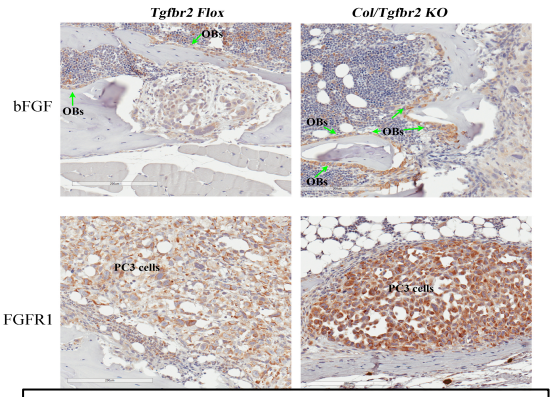

Figure 6. bFGF and FGFR1 expression in PC3 bone lesions of Col/Tgfbr2 KO and control mice.

3b. bFGF was not a mediator for *Col/Tgfbr2* KO induced LUCaP osteoblastic bone lesions (data not shown): This suggested the unique feature of osteoblastic PCa bone lesions compared to the osteolytic ones. We are currently investigating the downstream mediator for LUCaP bone lesions.

Task 4. Downstream mediators of TGF-β signaling in cells of the myeloid lineage.

4a. For promoting PC3 bone lesions: Cytokine array analysis showed the most up-regulated cytokines in the PC3 *LysM^{cre}/Tgfbr2* KO tibiae compared to the control tibiae were: IL-28, AXL, IL6R, IL10, HGF, IL-6, Fas ligand and Growth arrest specific 1. We are currently further investigating the specific role of these factors.

4b. For promoting LUCaP PCa dormancy: We found a critical piece of the *LysM^{cre}/Tgfbr2* KO mouse bone microenvironment is the expression of claudin-19 (Cldn19) in osteoblasts, in establishing a dormancy-permissive microenvironment for PCa cells. Claudins are the primary integral membrane proteins that form tight junctions, whose functions include forming cell adhesions, controlling cell permeability, and inhibiting cell proliferation. We have found that in order for Cldn19 to be expressed in osteoblasts, DNA demethylation is likely required. Thus, we **hypothesize** that loss of myeloid-specific TGF-β signaling activates Cldn19 expression in osteoblasts, leading to a growth-inhibitory bone microenvironment for PCa cells. A proposal for DOD idea award was submitted based on these exciting findings.

Therefore, we conclude that bFGF is a mediator for TGF-β signaling in osteoblasts on inducing osteolytic, but not osteoblastic PCa bone lesion development; Cldn19 expression in osteoblasts sets up a dormancy permissive bone microenvironment for osteoblastic PCa cells.

To determine whether the downstream mediators of cell specific TGF-β signaling in the bone microenvironment can be targeted for reducing PCa bone lesions (months 20-24):

Task 5. bFGF targeting for the increased PC3 induced bone lesions by *Col/Tgfbr2* KO: We found bFGF neutralizing antibody reduced the increased PC3 bone lesions by *Col/Tgfbr2* KO, while the recombinant bFGF protein promoted the PC3 bone lesions in the control mice to the level as in the *Col/Tgfbr2* KO mice (**Figure 7A, B&C**). These effects were correlated with respective changes in FGFR1, FGFR4, and p-AKT in the bone lesions (**Figure 7D**).

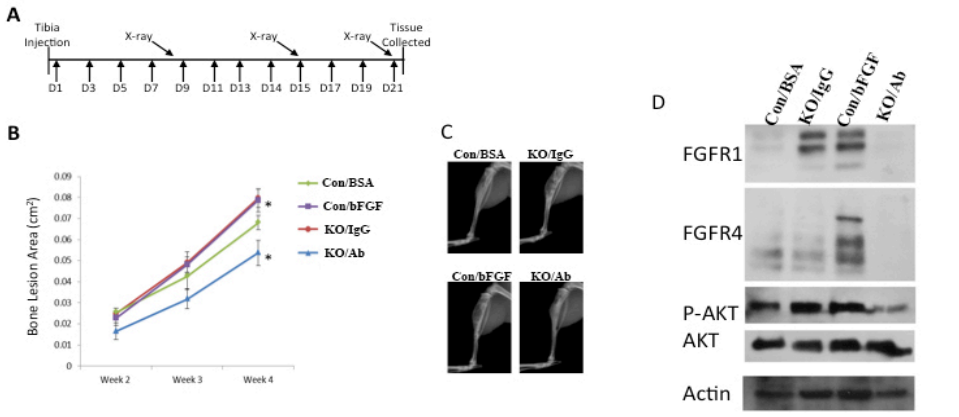

Figure 7. bFGF rescue experiments. A. bFGF recombinant protein or neutralizing antibody treatment schedule. B. Bone lesion measurements in different treatment group. C. Representative bone lesions from different group. D. Western blot showing respective changes in FGFRs and AKT pathway.

Thus, we conclude that bFGF is a functional mediator for osteoblastic TGF-β signaling in PC3 bone lesion development. bFGF binds to FGFR1 and FGFR4 to regulate downstream AKT signaling.

Key Research Accomplishments:

1. We found that TGF-β signaling in osteoblasts inhibited, but in myeloid promotes PCa-induced bone lesion development.
2. We determined that the inhibited PC3 bone lesions by TGF-β signaling in osteoblasts were correlated with decreased tumor cell proliferation, angiogenesis and osteoclastogenesis in the bone.
3. We identified bFGF as a functional downstream mediator of the effect of osteoblastic TGF-β signaling on PCa-induced osteolytic bone lesion development.
4. We are determining the downstream mediators for TGF-β signaling in myeloid lineage cells effects on PC3 bone lesions.
5. We found that the blocking TGF-β signaling only in myeloid lineage cells confers a growth-inhibitory bone microenvironment promoting LUCaP dormancy. Cldn19 is critical for this bone microenvironment. We proposed a newD DOD idea award for further investigating the mechanism.

Conclusions:

1. This sponsored research is on going with new direction based on our findings.
2. TGF-β signaling in cells of the osteoblast lineage inhibits, but in cells of the myeloid lineage promotes PCa induced bone lesions.
3. bFGF, mediates TGF-β signaling in the osteoblast lineage reducing PC3-induced osteolytic bone lesion development. ---manuscript in preparation
4. Blocking TGF-β signaling only in the myeloid lineage confers a growth-inhibitory bone microenvironment promoting LUCaP dormancy, likely through Cldn19. ---Proposal submitted

Reportable outcomes:

1. Sourik S. Ganguly, Xiaohong Li and Cindy K. Miranti. (2014) The host microenvironment influences prostate cancer invasion, systemic spread, bone colonization, and osteoblastic metastasis. Front. Oncol., 4: 364-379. http://www.ncbi.nlm.nih.gov/pubmed/25566502

2. Abstract for 2015 Cold Spring Harbor Laboratory Conference: Biology of Cancer: Microenvironment, Metastasis & Therapeutics.

3. Xiangqi Meng, Paul Daft, Alexandra Vander Ark, Jie Wang, Xiaohong Li. (2015) Osteoblast TGF-β signaling mediated basic-FGF inhibits prostate cancer bone metastasis. *Manuscript in preparation*

4. Sumbition of DOD idea award application on September 24, 2015.

References:

Li X, Sterling JA, Fan KH, Vessella RL, Shyr Y, Hayward SW, Matrisian LM, Bhowmick NA, 2012. Loss of TGF-beta responsiveness in prostate stromal cells alters chemokine levels and facilitates the development of mixed osteoblastic/osteolytic bone lesions. *Mol Cancer Res,* 10:494-503. http://www.ncbi.nlm.nih.gov/pubmed/22290877

Meng X, Vander Ark A, Lee P, Hostetter G, Bhowmick NA, Matrisian LM, Williams BO, Miranti CK, Li X, 2015. Myeloid-specific TGF-beta signaling in bone promotes basic-FGF and breast cancer bone metastasis. *Oncogene,* http://www.ncbi.nlm.nih.gov/pubmed/26279296

Morgan TM, Lange PH, Porter MP, Lin DW, Ellis WJ, Gallaher IS, Vessella RL, 2009. Disseminated tumor cells in prostate cancer patients after radical prostatectomy and without evidence of disease predicts biochemical recurrence. *Clin Cancer Res,* 15:677-83. http://www.ncbi.nlm.nih.gov/pubmed/19147774

Siegel R, Ma J, Zou Z, Jemal A, 2014. Cancer statistics, 2014. *CA Cancer J Clin,* 64:9-29. http://www.ncbi.nlm.nih.gov/pubmed/24399786

Appendices

None